Development and Validation of Flow Cytometry Assays for Autologous and Allogenic CAR T in Global Clinical Programs

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Introduction

There has been a breakthrough in cell & gene therapies, such as chimeric antigen receptor (CAR) T cell therapy. Most current CAR T cell therapies use patient's own engineered T cells, i.e., autologous CAR T cells. Due to the reduced cost and "off-the-shelf" applicability, more and more clinical trials are performed using the next generation allogenic CAR T cells, which are engineered T cells from healthy donors. Detection of CAR T cells in patients can be done via PCR or flow cytometry, however, flow cytometry has the advantage of being able to determine the phenotype of CAR T cells. As it is critical to assess CAR T cell expansion and persistence in patients as recommended by the FDA draft guidance, flow cytometry plays a central role in monitoring CAR T cells in clinical trials. Flow cytometry can support investigators to better understand how CAR T cells behave post-infusion and how they impact the patient's endogenous immunity.

CAR T therapy

Chimeric antigen receptor (CAR) T cell therapy is an emerging immunotherapy whereby T cells are genetically modified to recognize a particular antigen expressed by tumor cells. The engineered T cells express an artificial receptor consisting of an extracellular antigen recognition domain, typically a single chain variable fragment (scFv), a transmembrane domain, a co-stimulatory domain and an intracellular signaling domain. T cells are transduced (usually with a viral vector) to introduce the CAR construct. The resulting CAR T cells are then expanded *ex vivo* and infused back into the patient. CAR T cells identify their target on tumor cells and kill them by means of their cytotoxic potential. CAR T cell therapy has received commercialization approval from both FDA and EMA for the treatment of B cell malignancies. Most approved products use anti-CD19 or anti-BCMA CAR T cells.

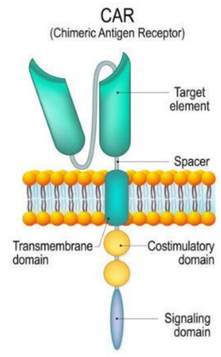


Figure 1: Schematic representation of a CAR. (obtained from ref: https://wuxibiology.com/solutions/therapeutic-modalities/car-t-cell-therapy/)

Flow cytometry and CAR T cell therapy

Flow cytometry is a powerful tool for comprehensive profiling of individual CAR T cells at multiple stages of clinical development, from product characterization during manufacturing to longitudinal evaluation of the infused product in patients. Post-infusion monitoring of CAR T cells includes the enumeration and phenotypic characterization of CAR T cells over time. Because infused CAR T cells are capable of activation, proliferation, maturation, and long-term persistence, their phenotypic characterization includes markers for differentiation, activation, exhaustion and senescence. As flow cytometry assays are used in all phases of CAR T therapy, the approach for assay development and validation depends on context of use of the data, the intended use specimen and regulatory requirements.

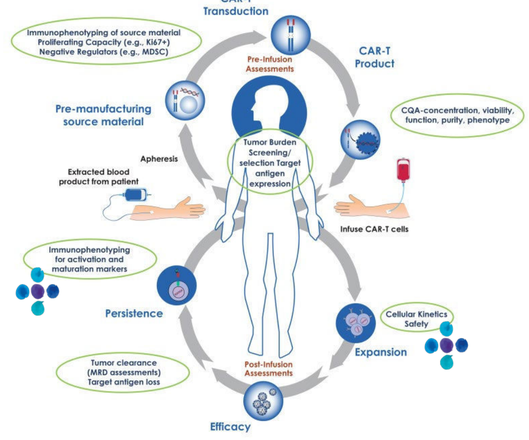


Figure 2: Flow cytometric evaluation supporting CAR T cell therapy. Flow cytometry is used throughout the lifecycle of CAR T cell therapies, from manufacturing (top circle) through post-infusion (bottom circle). Steps where Cerba Research develops assays are indicated with the Cerba Research logo. (obtained from ref: https://doi.org/10.1002/cyto.b.21985)

Autologous vs allogenic CAR T

Initial CAR T cell products were constructed with autologous cells, but more recently, allogenic CAR T therapies have also been developed. For **autologous CAR T therapies**, T cells are derived from the individual patient and engineered to express a specific CAR based on the disease target. For **allogenic CAR T therapies,** T cells are derived from healthy volunteers, engineered to express a specific CAR based on the disease target, and modified so the T cells are not rejected by the patient.

Manufacturing process	Personalized, complex logistics	Large-scale, Industrialized, standardized
Timelines	Long as to isolate, activate and gene modify before infusion	Immediate availability
Product characteristics	T cell characteristics depend on individual patients	T cell characteristics depend on the healthy donor, but are cryopreserved in batches to treat many different patients
Main issues/risks	Delay in treatment could lead to exacerbation of the disease	Graft versus host disease, rapid elimination by host immune system
Persistence	Intermediate to long (months to years)	Short to intermediate (weeks to months)
Redosing	Limited by number of cells	Possible, not limited by number of cells
Cost	High cost	Moderate cost

Figure 3: Comparison between autologous and allogenic CAR T therapy.

Clinical CAR T assay design - considerations

Panel design for a CAR T assay has to follow the current best practices for flow cytometric method design with regards to antigen fluorophore pairing, reagent titration and population spreading. A CAR T assay is well designed when it's able to report an accurate set of data for its intended use. Another aspect of assay development is the selection of reagents. The most critical reagent in a CAR T cell assay is the **reagent for CAR detection**. There are a variety of reagents available such as anti-idiotype monoclonal antibodies (mAb), target fusion proteins and anti-tag mAb.

Given that **anti-idiotype mAb** have a high affinity for CAR and exhibit a low nonspecific binding, they are highly recommended. A major drawback is that these reagents require a customized development. The combination of a long manufacturing period, the risk for variability between different lots and unpredictable elements within the sample forecast makes it challenging to manage reagent stock for global trials. Therefore, commercial antibodies, such as target fusion proteins or anti-tag mAb, are operationally seen as an easier alternative. **Target fusion proteins** bind the antigen-binding site of the CAR construct. They have a high specificity but a lower affinity for CAR compared to anti-idiotype mAb. **Anti-tag mAb** bind to a tag in the CAR construct, therefore, they are only applicable to CAR T cells with a tag built into the construct design.

Anti-idiotype mAb	Target fusion protein	Anti- Tag mAb
Binds idiotype of CAR	Binds ScFv of CAR construct	Binds tag
High affinity (sensitivity!!) high specificity	High specificity	Need for a unique tag
Non-commercially; cost	Lower affinity compared to anti-idiotype	Applicable only when CAR+ T has tag construct

Figure 4: CAR T detection approaches. Variety of detection reagents available for CAR T measurement with their advantages and limitations.

Additionally, it is vital to **avoid risks of interference** with antibody-based therapies - such as bispecific antibodies, anti-target or immune checkpoint therapy - targeting markers of interest in the assay, for example anti-CD38 or PD1/PDL1 inhibitors. This only applies to additional exploratory markers in the panel and not to the CAR detection itself. Clones of reagents used in the assays are to be selected based on their compatibility with the treatment of the patients. It is important that the selected clones bind to a different epitope of the antigen than the drug.

A unique challenge to flow cytometric assays is the detection of circulating CAR T cells that are present in low levels at certain time points or that the level of CAR expression may be down-regulated over time. Thus, the assay must be designed to robustly **detect rare events as well as dim antigens**.

Another challenge is the **lymphodepletion** of patients before infusing the CAR T cells. As allogenic CAR T therapy generally requires more intense lymphodepletion than autologous CAR T therapy, enrichment of the specimen is usually performed. Two available enrichment steps utilized at Cerba Research for patient sample testing are peripheral blood mononuclear cell (PBMC) isolation and the use of a bulk-lysis procedure.

Challenges for validating autologous and allogenic CAR T cell assays

Clinical validation of pharmacokinetic (PK) and CAR T characterization flow cytometry assays is challenging due to the lack of CAR positive control samples prior to the start of the trial. There are multiple solutions available, but the decision on which approach to follow depends on the type of assay and the intended use of the data.

To define the precision of the assay, **frozen CAR T cells should be spiked into the validation specimen**. To assess performance to monitor activation or exhaustion markers that are not present in healthy or disease donor samples, it can be opted to spike a cell line or commercially available control material. Frequently used control material is BD™ multi-Check, Veri-Cells™ Activated PBMC or fully customized TruCytes™ from Slingshot Biosciences.

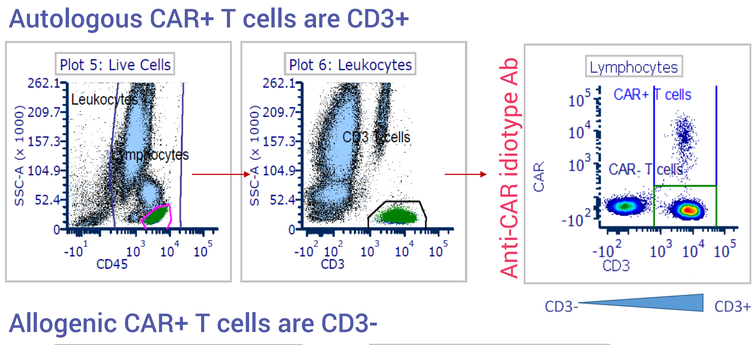
Spiking is not applicable for testing **performance correlation** between labs due to the variability of the matrix (blood and bone marrow aspirate (BMA)) and shipping/freeze/thaw in different labs. As no standard CAR+ control material is available, a set of primary reportables present in donor samples or QC material must be selected to conclude on the transparency between labs.

Stability assessment on specimen with spiked frozen CAR T cells will not provide accurate sample stability for CAR T cells in patients. Therefore, it is recommended to perform sample stability with patient samples during the CAR T cell expansion phase.

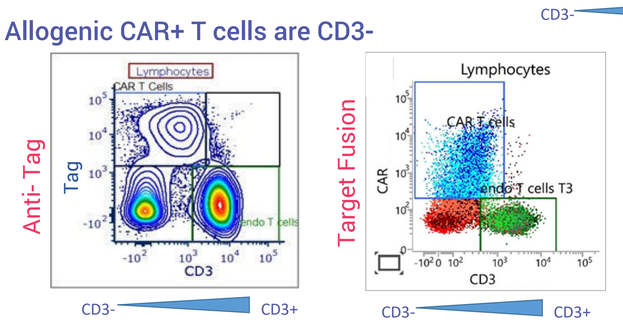
Implementation of both autologous and allogenic CAR T assays at Cerba Research

At Cerba Research, we have successfully validated and implemented flow cytometry assays on a global scale for both autologous and allogenic CAR T cell therapy trials.

Autologous CAR+ T cells are CD3+



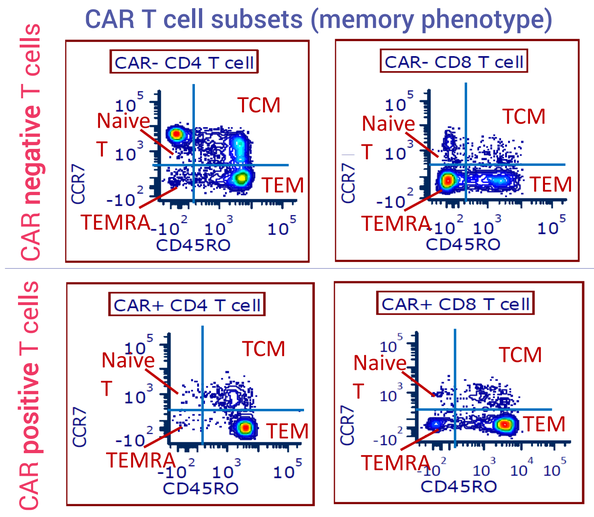
Allogenic CAR+ T cells are CD3-



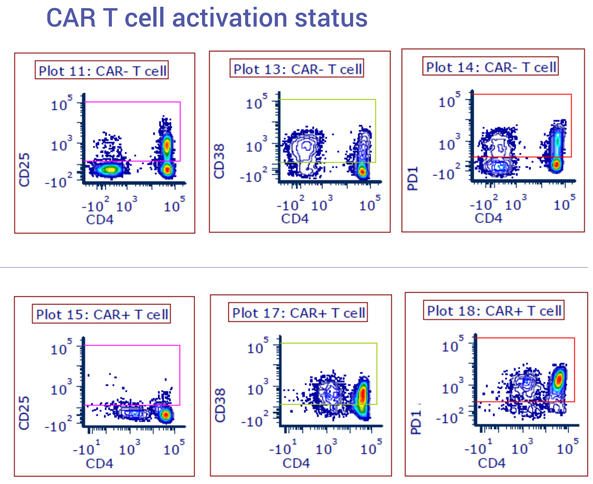
An aspect to keep in mind when designing the **gating strategy** is the character of the CAR T cell product it, whether it is autologous or allogenic. As autologous CAR T cells originate from the patient itself, gating can be achieved through CD3 positive T cell selection. Whereas allogenic CAR T cells are genetically knocked out for the T cell receptor (TCR) to reduce graft versus host risks, so CD3 is not expressed and require another gating strategy.

CAR positive T cells show a different profile for memory phenotype and activation markers than patient's endogenous T cells. Therefore, it is advisable to base gating on CAR negative T cells and adopt for CAR positive T cells.

CAR T cell subsets (memory phenotype)



CAR T cell activation status



Conclusion

Autologous and allogenic CAR T cell therapies have distinctive advantages and disadvantages. Several autologous therapies are approved by regulatory agencies, allogenic therapies are still at their clinical trial phase. At Cerba Research, we have successfully validated and implemented flow cytometry assays for both autologous and allogenic CAR T cell therapies for global trials. Cerba Research has the scientific expertise in house to develop an assay to meet the specific requirements for any CAR T clinical trial. Bulk-lysis procedure or PBMC isolation and specific gating strategy were implemented for allogenic CAR T trials. In addition, a CAR T enumeration assay was implemented globally for secondary endpoint assessment to measure absolute count of CAR T cells in patients.

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